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Extending Lifespan Using Various Prolongevity Interventions And Their Effects On Enhancing Dna Repair Activity

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EXTENDING LIFESPAN USING VARIOUS PROLONGEVITY INTERVENTIONS AND THEIR EFFECTS ON ENHANCING DNA REPAIR ACTIVITY

by

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DEDICATION

I would like to dedicate this thesis to my loving and supportive husband and son.

I am very thankful for their encouragement and support for the past 5 years.

ACKNOWLEDGEMENTS

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CHAPTER 1

Introduction

Aging is a time-dependent decrease in the physiological functions of cells, tissues, and organs (1). Aging is associated with a host of biological changes that contribute to a progressive decline in cognitive and physical function, ultimately leading to a loss of independence, and increased risk of mortality (2). Aging may be characterized by rising disability, frailty, and morbidity, heading to eventual death (3). Different theories attempt to explain aging molecularly by describing the impact of inflammation leading to disease, the accumulation of metabolic by-products, or DNA damage (1, 3). With increasing knowledge regarding aging and genomic stability, many studies have been performed in order to successfully find the link between various strategies and their effects on extension of lifespan.

A. Aging

A decreased ability to cope with stress is one of the hallmarks of aging (4). Aging is not a disease in its self; however it has been accepted that many diseases are associated with age (3). Aging is characterized by an exponential increase of oxidatively damaged proteins (2). It has been shown that lifespan can be extended in mice by genetic, dietary, and pharmacological interventions (5). Currently, it is generally accepted that continuing accumulation of damaged or aggregate macromolecules in somatic organs/ tissues is an underlying mechanism driving the aging process and is also presumed to be an overlapping factor in the etiology of many age-associated diseases (2, 6). By delaying the effects of aging, many years of research indicate that

diseases associated with aging are reduced by prolongetivity interventions such as reductions in caloric intake and mice genetically deficient for growth factors (6).

B. Mammalian Target of Rapamycin

The mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine protein kinase and a central controller of cell growth and metabolism in response to nutrients, growth factors cellular energy, and stress (7, 8). It should be noted that mTOR is now also used officially as abbreviation for 'mechanistic TOR' (1). mTOR has been found to play an important role in public health. mTOR was first discovered during the 1970s, when it was first isolated from a bacterial strain *Streptomyces hygroscopicus,* from the Rapa Nui Easter Island soil (9, 10). The activity of mTOR is controlled by amino acids, like leucine, one of the branched chain amino acids, in addition to growth factors and the overall energy supply through the AMP (adenosine monophosphate)-activated protein kinase (8). mTOR is the catalytic subunit of two distinct complexes called mTOR complex 1 (mTORC1) and mTORC2 in mammals (1, 7, 11). mTORC1 is composed of regulatory-associated protein of mTOR (RAPTOR), mammalian lethal with SEC13 protein 8 (mLST8), and mTOR and is rapamycin sensitive. A main function of the mTORC1 pathway is to regulate the accumulation of cell mass by activating mRNA translation and ribosome biogenesis and by inhibiting autophagy (13, 14). mTORC2, consisting of rictor, mammalian stress-activated protein kinase interacting protein 1 (mSIN1), proline rich 5 (renal) (PRR5), mLST8, and mTOR, is rapamycin insensitive (1, 7, 11, 12, 13). mTORC1 and mTORC2 share mLST8 and the recently identified DEP domain-containing mTOR-interacting protein (DEPTOR), which function as positive and negative regulators, respectively (1).

Biochemical and structural evidence suggests that both mTORC1 and mTORC2 may exist as dimers (1). mTOR contains a kinase domain near its C-termini, most closely related to the phosphatidylinositol 3-kinase (PI3K)-related kinase family, which includes ATM, ATR, and DNA-PK kinases (8).

In normal conditions, AKT controls the cell proliferation and endurance, as well as controlling nutrient metabolisms by the proliferation of some proteins (8, 10). A major target of TOR regulation in all cells is mRNA translation; in mammalian cells, mTOR stimulates translational initiation through the phosphorylation of eukaryotic initiation factor 4E (eIF4E) binding protein (4E-BP), an inhibitor of the binding of the mRNA-cap binding protein, eIF-4E, to the eIF-4G scaffold (1, 8). The phosphorylation of 4E-BP promotes its dissociation from eIF-4E enabling recruitment of the latter into the eIF-4F complex (1, 8). mTOR also directly phosphorylates and activates p70 S6 kinase (p70S6k); the latter regulates cell size and also phosphorylates the 40S ribosomal protein (1, 8, 13). Figure 1-1 illustrates the mTOR signaling network (7).
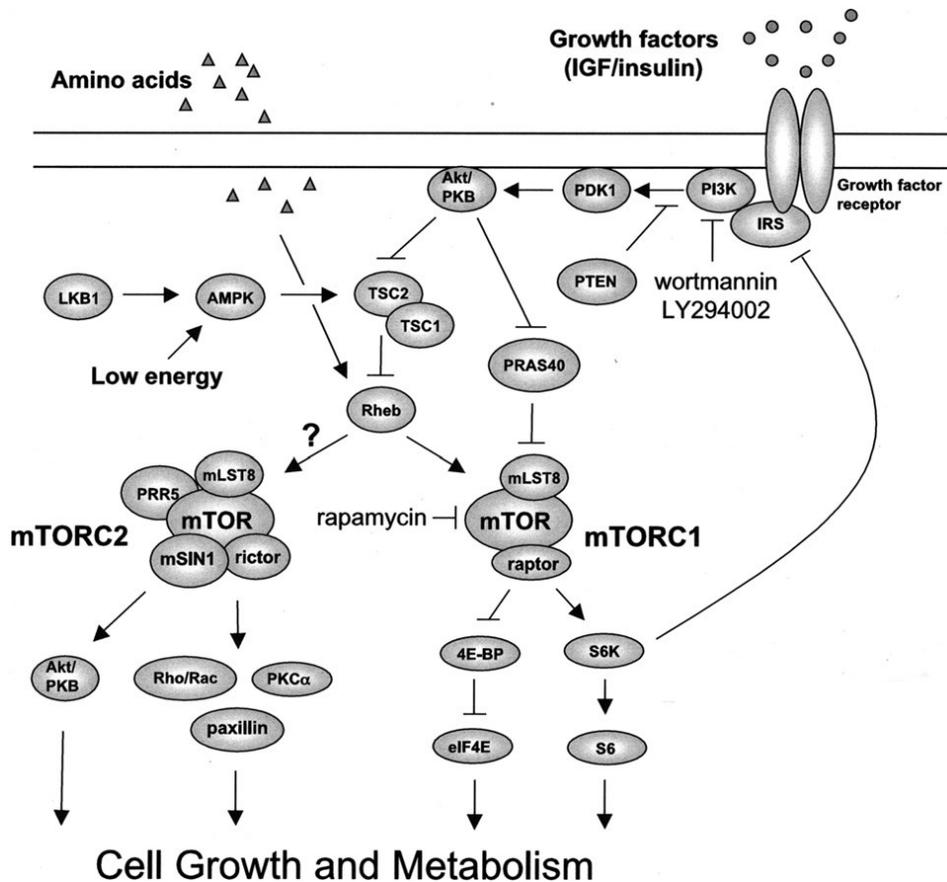
Figure 1-1. The mTOR Signaling Network

1. Rapamycin and Aging

mTOR was first discovered in the early 1990s in studies into the mechanism of action of rapamycin (also known as siromilus), which is a macrolide that was originally found as an antifungal agent and was later recognized as having immunosuppressive and anticancer properties (9, 14). Rapamycin and its analogues are already approved for human use as immunosuppressants and for advanced renal carcinoma (13). Rapamycin immunosuppressant actions result from the inhibition of T and B cell proliferation through the same mechanisms that rapamycin blocks cancer cell proliferation (1, 15, 16). Its immunosuppressant properties have been used often alone

or in combination with cyclosporine A after organ transplant (14). In yeast and mammals, rapamycin inhibits the ability of mTORC1, but not mTORC2, to phosphorylate its substrates (1, 7). Rapamycin might inhibit mTORC1 by disassociating RAPTOR from mTOR, thus preventing the access of mTOR to some substrates (1).

Overtime, the cumulative action of metabolic by-products, ionizing radiation, and exogenous chemicals damage and degrade cellular functions (1). Experimental evidence verifies that these types of timely changes include nearly all of the mTOR-regulated processes in aging (1). mTORC1 controls aging through several of its downstream processes including protein synthesis, transcription/translation, ribosome biogenesis autophagy, and mitochondrial activity (16). As an alternative to genetic interventions in modulating aging, the TORC1 branch of the TOR pathway can be successfully inhibited pharmacologically by rapamycin (13). Inhibition of mTORC1 may counter these sources of damage, enhance repair mechanisms, and may result in maintenance of healthy cells as seen in Figure 1-2 (1, 11, 13, 16, 17).
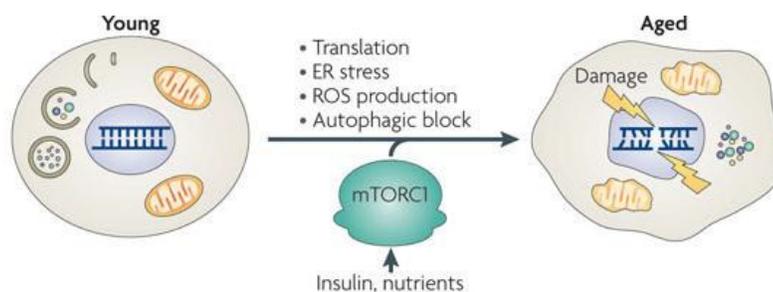
Figure 1-2. Aging in mTOR

An accumulation of ER stress, ROS production, and autophagic blocks can lead to the translation of in incorrect proteins, leading to DNA damage and hence promote aging. (1)

In recent years, the manipulation of nutrient sensing and stress response pathways has extended the lifespans of organisms from yeast to mammals (1, 13). Since mTOR is involved in translation, it has been found that reducing mRNA translation by genetic methods extends the lifespan of yeast, worms, flies, and mice (1, 18, 19, 20, 21). The rationale behind these results is that growth-promoting programs may accelerate aging by generating metabolic by-products and by directly inhibiting the clearance of these by-products (1, 18). Reduced translation may place smaller demands on the protein folding systems, thus decreasing the potential of misfolded protein aggregates as by-products (1). The idea that rapamycin extends mouse lifespan principally by blocking tumor development is consistent with studies showing rapamycin-mediated growth inhibition of many forms of cancer (19).

The National Institute on Aging Intervention Testing program (ITP) has previously reported significant increases in lifespan by rapamycin in both male and female heterogenous mice, and include parallel replication of protocols at three sites: the University of Texas (UT), University of Michigan (UM), and The Jackson Laboratory (TJL) (20). The effects were seen at each site evaluated independently (20). Rapamycin led to an increase in the fraction of mice alive at the 90th percentile survival age, taken as an index of maximal lifespan (20). It was reported that male and female mice given rapamycin from 20 months of age had longer lifespans than controls, and that rapamycin administered from 9 months of age led to lower mortality when evaluated at the median age for control survival (20). Thus, it was concluded that the life-extending effect of rapamycin is more pronounced when treatment starts earlier in life (21).

C. Calorie Restriction

Over the past 20 years, calorie restriction (CR), an experimental mode in which test animals receive a lower-calorie diet than *ad libitum*-fed controls, has emerged as the most potent, broadly acting dietary intervention for preventing carcinogenesis (22). CR without malnutrition is the most potent and reproducible physiological intervention for increasing lifespan and protecting against cancer in mammals (23). The finding that restriction of energy intake below the amount required for weight maintenance can slow the aging process and markedly extend lifespan was one of the most important health-related scientific discoveries of the 20th century (24). CR, which means to limit nutrient intake below normal levels without reaching malnutrition, has been shown to have many advantages; of them include the delay of many age-associated traits and diseases (25). CR also reduces the levels of many anabolic hormones, inflammatory cytokines, growth factors, reduces oxidative stress and cell proliferation, enhance autophagy and several DNA processes (23). CR diet provides the essential nutrients and vitamins but limits total energy intake of the animal, usually by 20-40% to *ad-libitum* fed controls (22, 25). In 1909, Moreschi was the first to publish that caloric restriction inhibits the growth of tumors transplanted in mice (23). While lifespan extension by food restriction appears to be due to alterations in aging processes, the underlying mechanism(s) by which food restriction exerts its anti-aging effects remain elusive (26).

CR, or lack of nutrients, activates 5' AMP-activated protein kinase (AMPK) and nicotinamide adenine dinucleotide (NAD +) dependent deactylases called sirtuins, or SIRT1 (27). Sirtuins, along with its activator resveratrol, can extend lifespan by antagonizing the mTOR/S6K pathway in part by AMPK activation (27). A decrease in

nutrients from CR may also deactivate mTOR through disabling the insulin/PI-3K pathway. Since it has been suggested that sirtuins and TOR may be involved in the same longevity pathway, CR may increase longevity through activation of sirtuins or deactivation of TOR (27).

CR is the most renowned dietary intervention in aging research as it has been very successful in the promotion of health and life extension in laboratory animals (26, 28). The majority of studies performed indicate that the age-related increase in oxidative damage to DNA is significantly reduced by CR (2, 26). Cabelof *et al*. (29) provides evidence that CR promotes genomic stability by increasing DNA repair capacity, specifically base excision repair (BER). They demonstrated that DNA polymerase β message, protein and activity are up-regulated by CR, indicating that DNA polymerase β, the rate-limiting enzyme in the BER pathway and a stress response gene, is the specific polymerase altered in response to CR (26, 29). More recently, adult-onset moderate 30% CR has been shown to reduce cancer morbidity and mortality in non-human primates, and a 50% reduced incidence of cancer in monkeys compared to controls (23). In rodents, 15-53% reduction in calorie intake below usual *ad libitum* intake caused a proportionate linear 20-62% reduction in tumor incidence (23). In mice, CR causes lifespan extension and changes in gene expression profile that are similar to those resulting from loss of S6K1, further supporting the view that CR acts through inhibition of mTORC1 pathway (1). Thus, findings from animal studies, including recent primate studies, suggest prolonged CR has the potential to extend health-span and thereby increase quality of life (2).

D. Crowded Litter

More recent evidence has shown that very short-term, transient nutrition restriction imposed prior to weaning by litter supplementation, crowded litter (CL), may increase mean and maximal lifespan in mice (5). The CL approach reduces food availability, but only in the first 3 weeks of life. In a recent study done by Steinbaugh M, et al., litter size was increased by 50% from 8 pups per mother to 12 pups per mother during the first 3 weeks of life. Mice in the enlarged litter group were found to have lowered levels of circulating insulin-like growth factor (IFG-I) at weaning and a small but lifelong reduction in body weight (5). Mutations associated with reduced action of growth hormone (GH) and/or IGF-I can increase lifespan by 40% or more, as seen in CL mice, as well as in young CR mice (5).

E. Health Consequences linked to Aging

Human aging is considered as one of the biggest risk factors for the development of multiple diseases such as cancer, type-2 diabetes, obesity, cardiovascular disease, and neurodegeneration (3, 30). It is also widely accepted that these age-related diseases result from a combination of various genetic, lifestyle, and environmental (23, 30). Functional decline is typically associated with the progressive loss of skeletal muscle mass and strength known as sarcopenia (muscle atrophy) (31). Different theories try to explain aging molecularly by describing the impact of inflammation leading to disease, oxidative stress, or the accumulation of metabolic by-products (1, 3, 27, 30). In addition to muscle atrophy secondary to age-related physiological changes, approximately four out of five older adults have at least one chronic health condition, and one out of two older adults have two or more chronic health conditions (31). Since

these age-related diseases are clearly exemplified to partake in processes involving inflammation and redox stress, it is conceivable that slowing down aging may come from the protection of these and other related diseases through mechanisms involving anti-inflammatories or recovery in redox stress (3, 30).

1. Obesity

Obesity is a condition is which excess or abnormal fat accumulation may present with adverse effects on health and decreased life expectancy (32). Recent estimates indicate that over two-thirds of persons aged 60 or older are overweight, and that one-third of adults aged 60 or older are obese (31). Compared to peers with a body mass index (BMI; kg/m^2) in the healthy range (20-24.9 kg/m^2), obese older adults with a BMI ≥ 30 kg/m^2 experience impairments in basic activities of daily living approximately five years earlier and are twice as likely to develop impairments in function and/or activities of daily living (31). Metabolic, hormonal and growth factor alterations associated with increased food consumption, decreased physical activity, and excessive adiposity, affect the regulation and expression of genes involved in DNA repair, cell proliferation and differentiation or apoptosis, allowing cells to accumulate damage and mutations, survive, proliferate and under permissive conditions, undergo malignant transformation (23). With aging, a decrease in muscle mass is typically observed, which is coupled with an increase in fat mass, most notably central adipose tissue (31). Accumulating evidence indicates that exercise- and/or CR-induced weight loss improves physical function, metabolic and hormonal alterations associated with excessive adiposity, among obese older adults (23, 31).

2. Diabetes

Type-2 diabetes results from insulin resistance, or in other words, the cells do not respond to the insulin that is produced by the pancreas. Similar to obesity, diabetes is highly prevalent among older adults, and currently afflicts more than one in five adults aged 65 years or older (31). Additionally, the prevalence of diabetes among older adults is rising and projected to reach 30% in the next three decades (31). Although obesity is a significant contributor to diabetes-related disability, there appears to be a distinct effect of diabetes on risk for disability independent of BMI (31). In contrast to obese older adults without diabetes, non-obese older adults with diabetes often display an accelerate loss of absolute muscle mass (31). As noted earlier, body fat redistribution occurs during aging and places older adults at increased risk for accumulation of abdominal fat, which contribute to unhealthy metabolic conditions and reductions in insulin sensitivity (31). Furthermore, high levels of visceral fat have been associated with simultaneously increases in production of the pro-inflammatory cytokines and decreases in production of anti-inflammatory cytokine, adiponectin (23, 31). Adiposity generally shows a direct linear relationship with serum insulin concentrations (23). The metabolic disturbances associated with obesity can therefore place older adults at increased risk of diabetes, and the subsequent glucose dysregulation and insulin resistance associated with diabetes may adversely affect appetite regulation and lead to excessive food intake (23, 31). An increased understanding of the mechanisms through which both obesity and diabetes interact to affect rates of sarcopenia and functional decline can help facilitate the development of targeted interventions specifically designed to improve the functional status of this high risk population (31).

3. Cardiovascular disease

There is growing evidence based on findings from animal studies and a limited number of human trials that CR has the potential to delay aging of the cardiovascular system and help prevent atherosclerotic cardiovascular disease (CVD) (24). The significance of this finding is obvious, since CVD remains the leading cause of mortality and one of the main causes for morbidity in older adults, despite tremendous overall progress in its prevention and treatment (1, 2, 13, 24). The classic risk factors for atherosclerotic diseases (cardiovascular, cerebrovascular, and peripheral vascular) include dyslipidemias (high low-density lipoprotein cholesterol [LDL-C], low high-density lipoprotein [HDL-C], and elevated triglycerides), elevated blood pressure, diabetes mellitus, and smoking (31). Additionally, diabetic patients often have accelerated progression of atherosclerosis, which can decrease peripheral blood flow, resulting in poor muscle perfusion (24). It has been found all of the latter mentioned risk factors, except smoking, are improved in response to CR in animal models, either directly or via weight loss associated with it (31), therefore reducing the risk for atherosclerosis.

4. Cancer

Cancer is a complex multistage disease associated with an accumulation of multiple DNA mutations that cause deregulation in cell proliferation, differentiation, and a loss of normal tissue organization, and eventually tissue invasion and dislocation to distant sites (metastasis) (23, 33) Developing cancer in the US has an extremely high probability with approximately 44% of men and 37% of women who can develop cancer at some point during their lifetime (34). The accumulation of multiple DNA mutations in critical genes (i.e. oncogenes or tumor suppressor genes) of particular cells, if not

properly controlled through induction of senescence or apoptosis, can lead to uncontrolled cell proliferation and progressive transformation of normal human cells into highly malignant tumor cells (22, 23). Epidemiological data on geographical and chronological variations in cancer incidence have shown that environmental factors have profound effects in the initiation, promotion, and progression of some of the most common cancers in Western countries (23). Various studies into the investigation of lifestyle factors and behaviors have led to the conclusion that the majority of cancer deaths in many developed countries can be attributed to factors such as unhealthy diets, tobacco, alcoholism, infections and occupational exposures (23). Aging is the highest risk factor for cancer (35). Significant evidence suggest that DNA damage (oxidative stress) has a role in the organismal aging process (35). DNA damage compounded with defects in DNA repair can lead to genomic instability and consequential tumorigenesis (35).

i. mTOR and Cancer

The mTOR pathway is believed to largely drive the malignant behavior of many types of tumors, and therefore mTOR inhibition is considered an attractive target for cancer (17, 36). Most dramatically, the TSC1(tuberous sclerosis 1, also known as harmartin) –TSC2 (also known as tuberin) tumor suppressor complex — the inactivation of which causes the tumor-prone syndrome tuberous sclerosis complex (TSC) and the related disease lymphangio leiomyomatosis (LAM) — has emerged as a key negative regulator of mTORC1 (14, 16). The upstream and downstream signaling components of mTORC1 are frequently altered in a number of human tumors (1, 9, 14) along with other regulators such as lipid phosphatase PTEN (phosphatase and tensin homolog), which

acts as a tumor suppressor gene (1,14, 16). A loss of PTEN function caused by a deletion or mutation can be seen in as many as 50% of all solid human tumors (14, 37). Similarly the p53 tumor suppressor gene is the most frequently mutated gene in human tumors (over 50%) and over 80% have poorly functional p53 signaling (38). Given the potent anti-proliferative effects of rapamycin, it is not surprising that the mTOR signaling pathway reg2ulates the levels of several proteins directly involved in controlling cell division as seen in figure 1-3 (16).
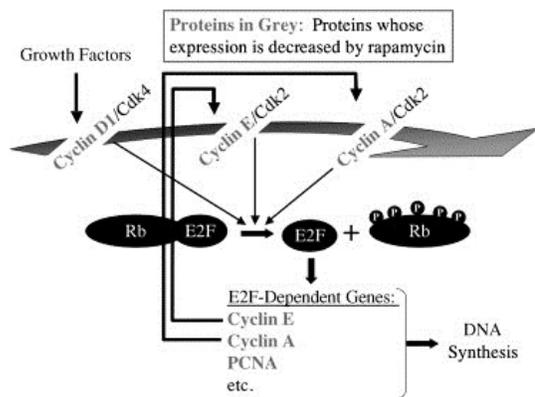
Figure 1-3. Rapamycin down-regulates proteins that drive cell proliferation (16).

ii.   Calorie Restriction and Cancer

Over the past 30 years, CR has emerged as the most potent, broadly acting dietary intervention for preventing carcinogenesis in rodent models of cancer (22). To date, there is consensus that excessive adiposity due to overconsumption of energy-dense foods and sedentary lifestyle increases the risk of developing cancer (23). Data have shown that CR without malnutrition inhibits spontaneous, chemically-induced and radiation-induced tumors in several animal models of cancer (23). The age when CR is started, the severity of CR, and the strain/genetic background of the animals determine

the magnitude of cancer prevention or delay (23). Many of the effects of CR are likely mediated by regulating gene expression including the up-regulation of tumor-suppressor genes, of genes promoting DNA and cellular repair, protein turnover, stress resistance, and anti-oxidant genes, and the down-regulation of pro-inflammatory genes and modulation of energy metabolism pathways (22, 23). Whether CR with adequate nutrition will reduce cancer incidence in humans is unknown, but data from studies of long-term CR in humans suggest that the metabolic and physiological responses to CR are similar to those in rodents and monkeys (23).

F. Oxidative Stress

Among the many factors contributing to aging, one of the highly investigated focuses on the theory that there is gradual decline of mitochondrial function with age leading to progressive tissue damage via oxidative stress (39). Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, so-called oxidants or reactive oxygen species (ROS), and their elimination by protective mechanisms, referred to as antioxidants (40, 41). Cellular damage induced by oxidative stress is a common consequence of the respiratory chain in the mitochondria (40, 41). Oxidative stress is caused by increased levels of reactive oxygen species (ROS) and decreased production of antioxidants (defense system) (35, 40, 43). Examples of such damaging agents (ROS) include: superoxide anion, hydroxyl radical, hydrogen peroxide, and organic peroxides (40, 43). ROS are a specific class of free radicals that are formed from molecular oxygen in biological systems (44). Drugs, hormones, and other xenobiotic chemicals can produce ROS by either direct or indirect mechanisms (44, 45). The distinguishing chemical property of radicals is the possession of an

unpaired electron (Heydari). This can cause harmful effects in the ability to damage nucleic acids (45). ROS can be generated in numerous ways, but most notably by a process termed mitophagy. This specific classification of autophagy is known as the breakdown of old or damaged mitochondria (40, 46). In addition, ROS can be formed as intermediates in various enzymatic reactions and many cell signaling pathways in the cell (Heydari).

Oxidative stress can result in critical impairment to various cells of an organism. Repeated damage and production of such species alter the structure and performance of the cell, which can lead to premature aging and reduced lifespan (39, 40, 41). Many illnesses, including cancer, have been associated with ROS. Prolonged DNA damage leads to serious problems such as induction of signal transduction pathways, arrest or induction of transcription, replication errors, and genomic instability (45). Hence, ROS may play a role in the survival rate of cancer patients (40, 44, 45). This is exemplified in a group of enzymes known as "nicotinamide adenine dinucleotide phosphate oxidase" (NOX), leading to the production of superoxide, which can positively affect cancerous proliferation and existence (40). It is important to highlight that ROS can harm or progress cancer in any of its stages, however it is most effective in the primary phase (40). Types of damage inflicted by ROS include point mutations, chromosomal breaks, and DNA cross-linking, which result in inactivation of tumor- suppressor genes (40, 44, 46). As a consequence, oxidative stress can cause complications by creating further stress and inducing mutations to existing cancerous lesions (40, 44).

Thus, a mechanism needs to be in place to manage such stress that can cause long term breakdowns. This antioxidant defense system is used to target and protect

the organism (40). It is not a single entity, but rather an elaborate type that includes superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione (GSH) (40, 47). Superoxide dismutase is the most important enzyme in regulating protection against ROS in the cell (47). It functions by converting superoxides into oxygen and hydrogen peroxide. While this does not completely eliminate the toxic effects, it helps to reduce the amount experienced in the cell (47). This is then followed by action of the enzyme catalase, finalizing the work started by SOD. It breaks down hydrogen peroxide to water and oxygen. Glutathione peroxidase employs peroxidase activity to decrease the levels of free hydrogen peroxides (47).

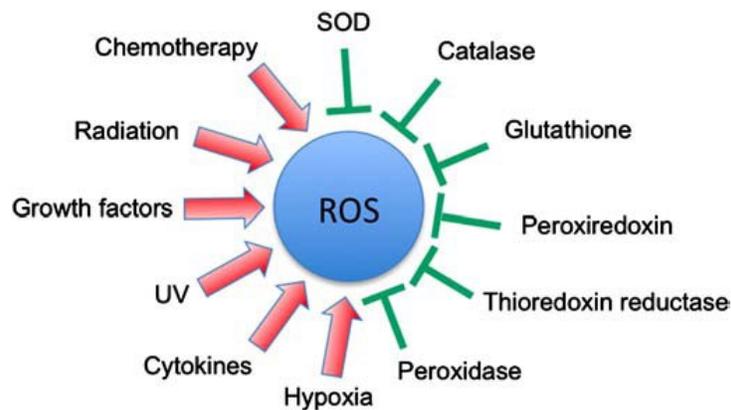**Figure 1-4.** Oxidative Stress and Reactive Oxygen Species (ROS) (40)

G. Base Excision Repair Pathway

The genome of eukaryotic cells is under continuous attack from a variety of DNA-damaging agents (endogenous and exogenous), which lead to many types of DNA lesions (single-strand breaks, double-strand breaks, base mismatches, intra- or interstrand cross-links) (35). To avoid the harmful consequences of damage

accumulation, multiple DNA repair pathways have evolved, each associated with specific class of lesions (35). The major source of endogenous DNA damage is ROS (free radicals) generated from normal cellular metabolism (35, 48). Various mechanisms exist to repair such damages, one of which is the *Base Excision Repair* (BER) pathway that repairs oxidative base lesions (35, 48).

Base excision repair pathway is the most common repair used to fix DNA damages that occur spontaneously or due to reactive oxygen species (35). It has been estimated to be responsible for the repair of as many as one million nucleotides per cell per day (49). ROS are constantly generated in living organisms as by-products of cellular metabolism, but can also be produced as a consequence of ionizing radiation, chemotherapeutic drugs and environmental exposure to transition metals and chemical oxidants (figure 1-4) (35). ROS, produced during oxidative stress, generate a variety of DNA lesions, resulting in genomic instability. Thus, it plays a predominant role in dealing with inappropriate base pairing, oxidation and alkylation of bases, base loss at specific sites, and chromosomal single-strand breaks that may result from attack by mutagens (35).

BER is a critical process for genomic maintenance, as highlighted by the severe phenotypes seen in animals deficient in BER function, including cancer, premature aging and metabolic defects (35). The initial step in BER uses DNA glycosylases, which cleave the *N*-glycosyl bond between the sugar and the base, thus releasing the damaged base to form an abasic site, also termed apurinic/apyrimidinic (AP) site (35, 48). Since the abasic sites are highly mutagenic, efficient DNA repair is critical. The AP sites are then cleaved by AP endonucleases (APE). The resulting single-strand break

can then be processed via two different routes: short-patch BER (single nucleotide replacement) or long-patch BER (more than one nucleotide replacement). Afterwards, the DNA β-polymerase employs its 5-deoxyribosephosphodiesterase activity to remove the 5' abasic site (the *N*-glycosidic bond linking nitrogenous base to the sugar moiety) and subsequent sealing of the gap (35). At this time, DNA polymerase is responsible for replacing the appropriate or complementary base (35). The final step of BER entails ligation of the remaining nick, by either Ligase 3-XRCC1 complex in short patch BER, or by Ligase 1 alone in long-patch BER (35).
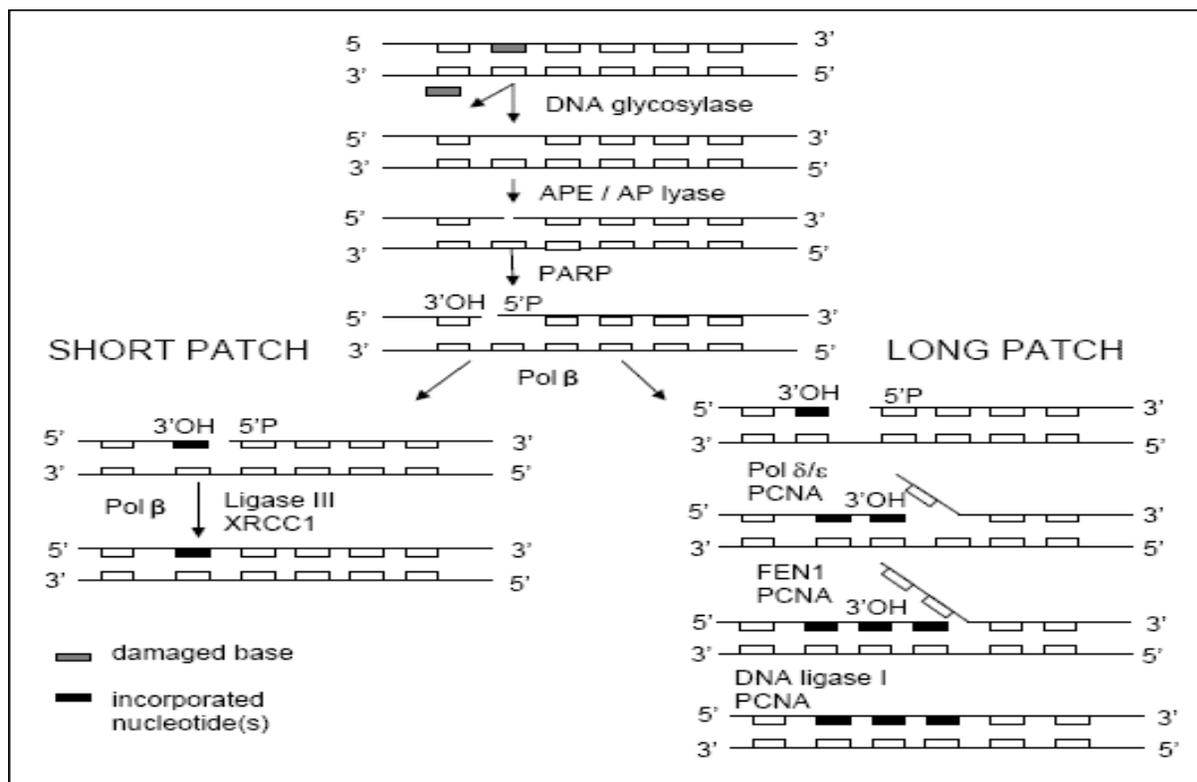
Figure 1-5. Base Excision Repair Pathway: The Short Patch and Long Patch

In the oxidative damage reduction hypothesis, CR has been demonstrated to reduce the accumulation of oxidative damage to biological molecules including DNA, protein and lipids in rodents (26). This reduction in oxidative damage is proposed to be

due to a decline in ROS, an enhancement of protective mechanisms, an increase in repair capacity, or a combination of all the aforementioned (26). The process in which the dietary intervention of CR diet acts to alter DNA repair capacity can be seen illustrated in figure 1-6.

MODULATION OF GENOMIC INSTABILITY
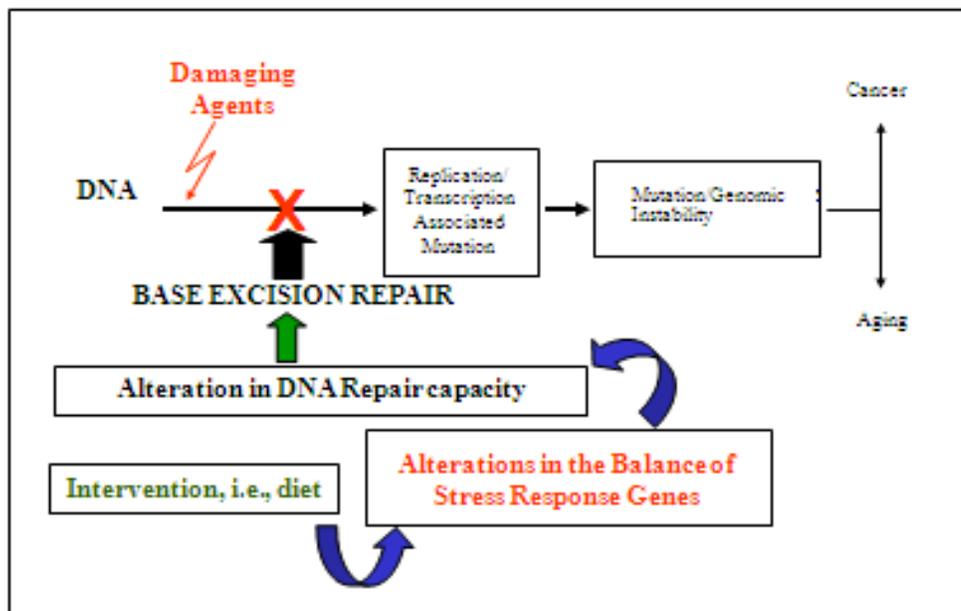
Figure 1-6. Modulation of Genomic Instability (Heydari lecture)

H. Folate and Folate Deficiency

Folic acid, an essential water soluble vitamin and cofactor in one-carbon metabolism, has been associated with the etiology of many chronic diseases such as cardiovascular disease, neurological degeneration, and cancer (49). Folate deficiency has been shown to enhance the potency of carcinogenic agents (49, 50, 51). Although the underlying mechanism of folate deficiency-induced tumorigenesis is largely unknown, many different factors have been suggested as being responsible for its

carcinogenic effect, one of which is DNA methylation (50). Folate deficiency enhances the carcinogenic effect of dimethylhydrazine, whereas folate supplementation is protective (51). The accumulation of strand breaks, mutations, and chromosomal instability observed in response to folate deficiency all suggest that DNA repair capacity inhibited (51).

A recent study was conducted by Cabelof *et al*. in young male mice that were randomly assigned to the control group receiving a folate adequate diet containing 2 mg/kg folic acid, or to the experimental group receiving a folate-deficient diet containing 0 mg/kg folic acid. The experimental diets continued for 8 weeks and the animals' food intake and body weights were monitored twice weekly (51). The objective of this study was to directly determine the effect of folate deficiency on BER capacity. As expected, a significant decrease in the level of serum folate was observed in the folate-deficient mouse (51). Since BER is shown to be DNA damage-inducible, BER is expected to be upregulated in response to increased levels of DNA damage that occur in folate deficiency. But surprisingly, no increase in BER activity was observed in response to folate deficiency (51). Moreover, DNA polymerase β is determined to be rate-determining in BER, however, it was shown that the lack of inducibility of BER in response to folate deficiency is preceded by a lack of induction in DNA polymerase β (51).

CHAPTER 2

Specific Aims

Understanding the impact that rapamycin, calorie restriction, and a crowded litter have on repair pathways in the cell is a vital process that can help elucidate the mechanism on life extension and carcinogenesis. By far, age is the biggest risk factor for a number of intrinsically driven diseases. For the past several years, many studies have been conducted with rapamycin supplemented diets and calorie restricted diets, both with results exhibiting the positive effects on the expansion of lifespan and decreased risk of carcinogenesis. Also, much emphasis has been elucidated on the relationship between folate restriction and the result of many diseases, such as cancer. Nevertheless, all of the aforementioned are topics of interest in current research, that if investigated even further can potentially be employed as an interventional means to decrease DNA damage (oxidative stress), and also increase the expression of repair genes in the cells. Using Real-Time PCR analysis for measuring mRNA (transcriptional) levels of specific oxidative stress and BER pathway genes in mice cells exposed to either a rapamycin diet, calorie restricted diet, or crowded litter placement, the indicated hypothesis was tested: the cells ability to overcome genomic damage is enhanced by a rapamycin diet, reduced calorie diet, and crowded litter placement, and positively impact base excision repair genes to extend lifespan, and ultimately reduce the risk of carcinogenesis. The following are the specific aims of the research:

<u>Specific Aim 1</u>. To examine the impact of rapamycin diet, calorie restricted diet, and crowded litter in important BER pathway genes: UNG, APE, B-POL, LIG III, XRCC1

<u>Specific Aim 2</u>. To examine if the expression of GADD45g, PARP, p53, and mTOR are altered in response to exposure to a rapamycin diet, calorie restricted diet, and crowded litter placement.

<u>Specific Aim 3</u>. To compare the expression of BER genes in response to folate status with that of the BER genes' expression in rapamycin diet, calorie restricted diet, and crowded litter placement.

CHAPTER 3

Methods

A. Animals

Experiments (52) were performed in specific pathogen-free mice produced at the Unit for Laboratory Animal Medicine, University of Michigan School of Medicine in Ann Arbor, Michigan. These mice were produced by mating CB6F1 females with C3D2F1 males to produce a genetically heterogenous population. All practices performed on animals were in agreement with the National Institutes of Health guidelines for the care and use of laboratory animals.

B. Diets and Treatment

Mice were randomly assigned to 1 of 4 groups which included: 4 control samples fed an *ad-libitum* diet, 4 rapamycin-treated samples, 4 crowded litter (CL) samples, and 5 calorie-restricted (CR) samples. All animals are a part of an ongoing current study at the University of Michigan. The CR mice were given 60% of the amount of food consumed by the control mice started at 6 weeks of age. The CL mice consisted of litters containing 10 or more pups, for the first 3 weeks of life. Rapamycin (from LC Labs, Woburn, MA) was microencapsulated by Southwest Research Institute in San Antonio, Texas, using a spinning disk atomization coating process with the enteric coating material Eudragit S100 (Röhm Pharma, Germany). This coating increased the fraction of rapamycin that survived the food preparation process by 3- to 4-fold, and protected the agent from digestion in the stomach (52). Encapsulated rapamycin was

then incorporated into 5LG6 mouse chow and distributed to the lab at University of Michigan. The rapamycin diet commenced for the mice between 4-6 weeks of age

C. Analysis

Figure 3-1 shows the date of birth, date of sacrifice, and weight at time of sacrifice. At 12-13 months of age, mice were anesthetized in a CO_2 chamber and sacrificed by cervical dislocation. Harvested liver, all male, was flash-frozen and stored in liquid nitrogen. Next, the liver tissues were fixed in 10% neutral buffered formalin, and trimmed to approximately 30 mg each. After obtaining the appropriate samples, RNA isolation and quantification, cDNA synthesis and purification, and Realtime PCR were performed.

1. Realtime PCR

Total RNAs were isolated from the liver tissue of mice, using the RNeasy Mini Kit (Qiagen, Valencia, CA) per manufacturer's protocol and Dr. Heydari. cDNAs were synthesized from 1 µg of RNA using random hexamer primers. cDNAs were purified with the QIAquick PCR Purification kit (Qiagen). The levels of cDNAs were quantified using a Lightcycler real time PCR machine (Stragagene, La Jolla, CA). PCR contained 3 µL of purified cDNA, 12.5 µL of qPCR master mix, and 0.5 µmol/liter each of sense and antisense primers (Roche). For all amplifications, PCR conditions consisted of an initial denaturing step at 95 ºC for 5 minutes followed by 40 cycles of denaturation at 95 ºC for 10 seconds, and annealing at 60 ºC for 30 seconds, with a melting curve analysis from 60 to 95 ºC to confirm specificity. External standards were prepared by amplification of cDNAs for each gene. The amplicons were cloned into pGEM-T Easy

vector, linearized with appropriate restriction enzyme, and used to prepare external standard curves. The level of each transcript was normalized to ribosomal protein L15 (*RPLO)*. *RPLO* is a housekeeping gene that is responsible for serving as a control for normalization of RNA levels in comparison to other genes. Results are expressed as mean values depending on number of animals per experimental group.

2. Statistical Analysis

Statistical significance between means was determined using the "unpaired t-test" as described previously (53). *P* values less than 0.05 were considered statistically significant.

	Sample	Gender	DOB	DOD	WT (g)
Control	H0469	M	12/2/2009	11/15/2010	45.9
Control	H0476	M	12/2/2009	11/15/2010	40
Control	H0777	M	12/3/2009	11/15/2010	46.6
Control	H0784	M	12/3/2009	11/15/2010	38.3
Rapa	H0252	M	11/7/2009	11/15/2010	45.7
Rapa	H0259	M	11/7/2009	11/15/2010	32.9
Rapa	H0707	M	12/3/2009	11/15/2010	51.5
Rapa	H0714	M	12/3/2009	11/15/2010	43.5
CL	H2149	M	1/22/2010	2/1/2011	33
CL	H2156	M	1/22/2010	2/1/2011	30.9
CL	H1463	M	1/5/2010	1/18/2011	46.7
CL	H1470	M	1/5/2010	1/18/2011	47
CR	H2891	M	2/10/2010	2/15/2011	34.8
CR	H2898	M	2/11/2010	2/15/2011	29
CR	H3108	M	2/23/2010	2/15/2011	32.3
CR	H3199	M	2/23/2010	3/1/2011	29.2
CR	H3206	M	2/23/2010	3/1/2011	32.9

Figure 3-1. Date of birth (DOB), date of death/sacrifice (DOD), and weight upon sacrifice.

CHAPTER 4

FIGURES

Figure 4-1. Impact of rapamycin diet, crowded litter, and calorie restriction on expression of UNG mRNA levels. UNG mRNA levels in the liver tissue of mice in each group were quantified using real-time PCR and normalized against RPLO. Values represent an average (±SEM) of data obtained from 4-5 mice in each group. Values with different letter superscripts indicate significant differences at $P < 0.05$. C, control; R, rapamycin-treated; CL, crowded litter; CR, calorie-restricted.

Figure 4-1

Figure 4-2. Impact of rapamycin diet, crowded litter, and calorie restriction on expression of APE mRNA levels. APE mRNA levels in the liver tissue of mice in each group were quantified using real-time PCR and normalized against RPLO. Values represent an average (±SEM) of data obtained from 4-5 mice in each group. Values with different letter superscripts indicate significant differences at $P < 0.05$. C, control; R, rapamycin-treated; CL, crowded litter; CR, calorie-restricted.
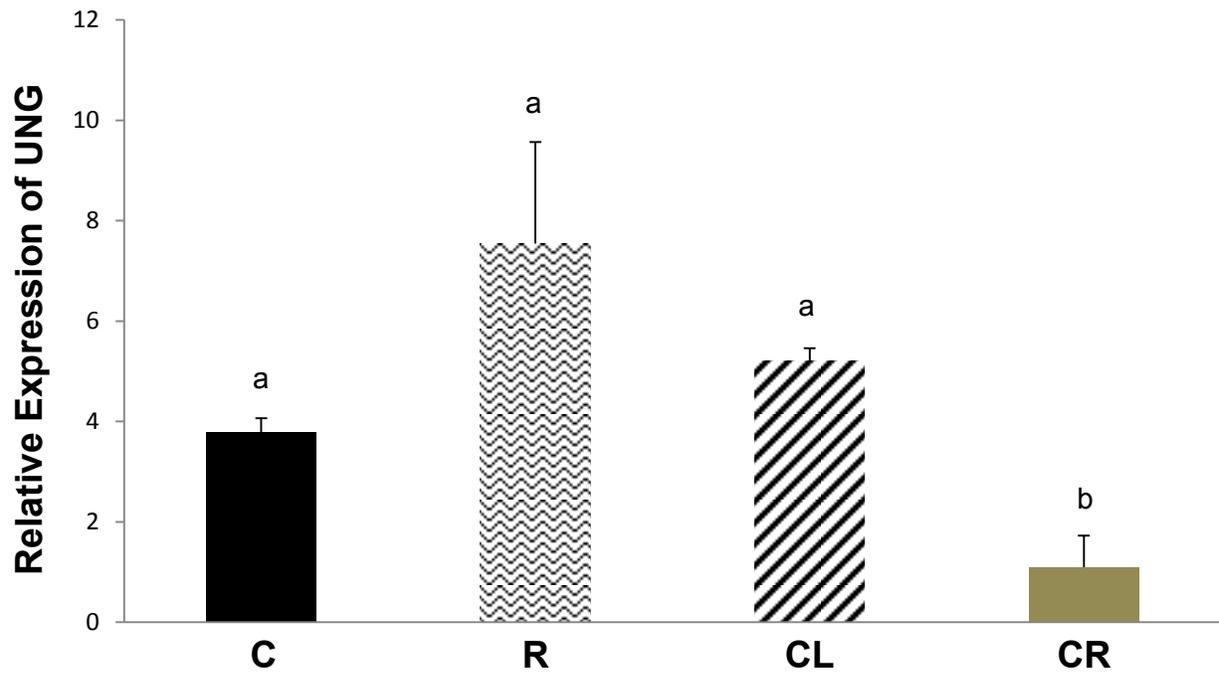
Figure 4-2

Figure 4-3. Impact of rapamycin diet, crowded litter, and calorie restriction on expression of β-pol mRNA levels. β-pol mRNA levels in the liver tissue of mice in each group were quantified using real-time PCR and normalized against RPLO. Values represent an average (±SEM) of data obtained from 4-5 mice in each group. Values with different letter superscripts indicate significant differences at $P < 0.05$. C, control; R, rapamycin-treated; CL, crowded litter; CR, calorie-restricted.
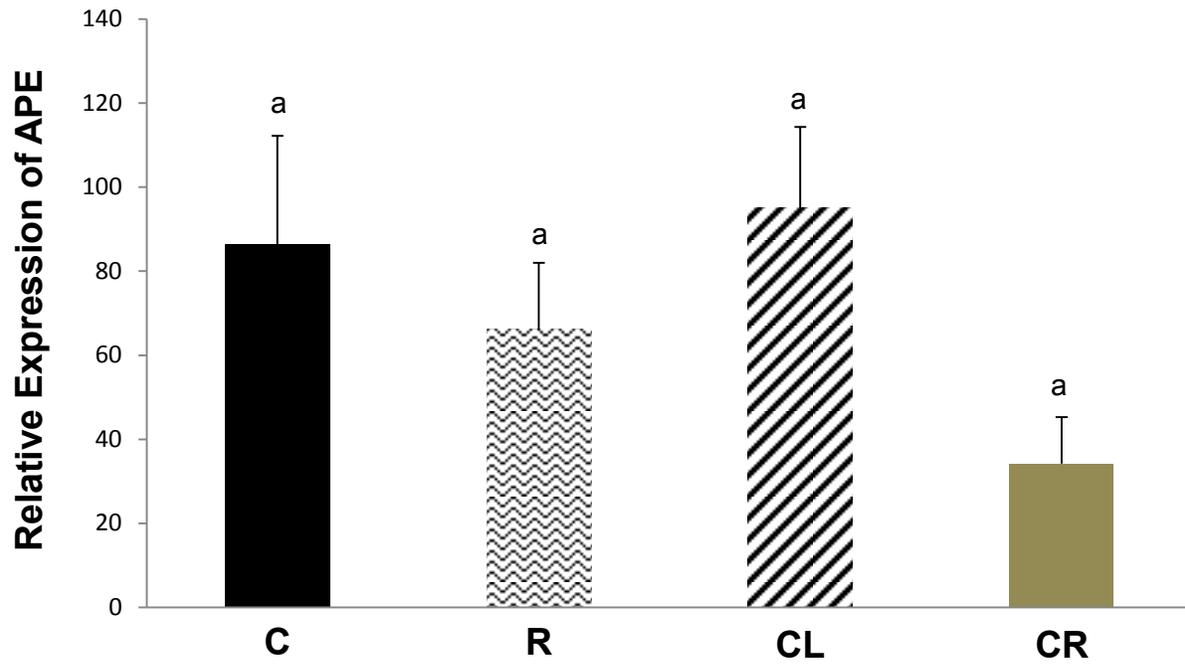
FIGURE 4-3

Figure 4-4. Impact of rapamycin diet, crowded litter, and calorie restriction on expression of LIG3 mRNA levels. LIG3 mRNA levels in the liver tissue of mice in each group were quantified using real-time PCR and normalized against RPLO. Values represent an average (±SEM) of data obtained from 4-5 mice in each group. Values with different letter superscripts indicate significant differences at $P < 0.05$. C, control; R, rapamycin-treated; CL, crowded litter; CR, calorie-restricted.

FIGURE 4-4

Figure 4-5. Impact of rapamycin diet, crowded litter, and calorie restriction on expression of XRCC1 mRNA levels. XRCC1 mRNA levels in the liver tissue of mice in each group were quantified using real-time PCR and normalized against RPLO. Values represent an average (±SEM) of data obtained from 4-5 mice in each group. Values with different letter superscripts indicate significant differences at $P < 0.05$. C, control; R, rapamycin-treated; CL, crowded litter; CR, calorie-restricted.
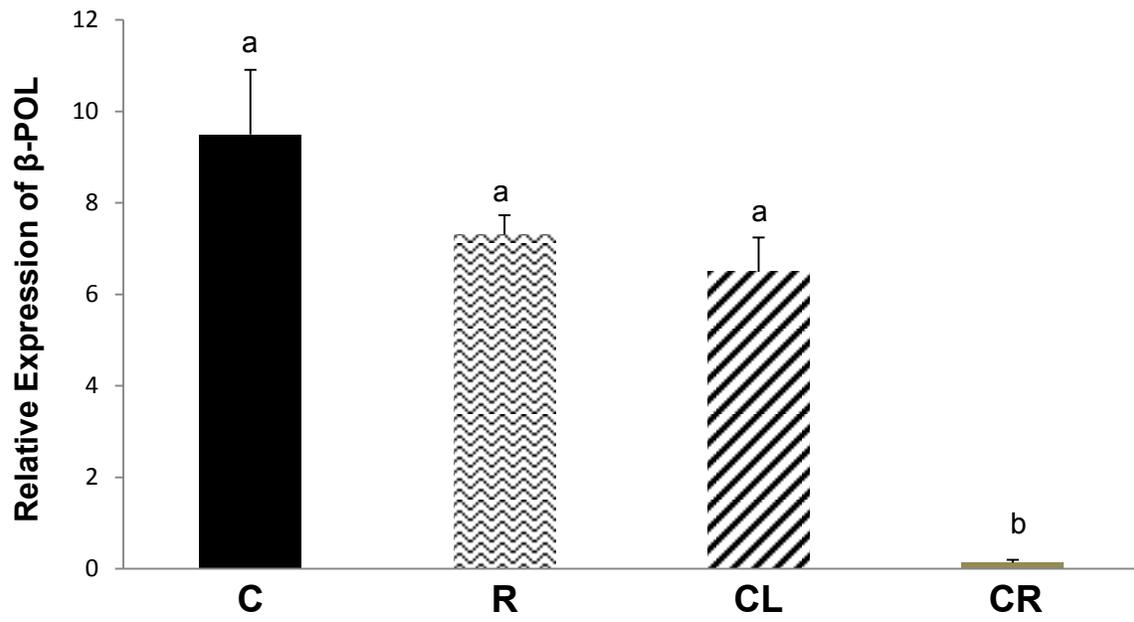
FIGURE 4-5

Figure 4-6. Impact of rapamycin diet, crowded litter, and calorie restriction on expression of p53 mRNA levels. P53 mRNA levels in the liver tissue of mice in each group were quantified using real-time PCR and normalized against RPLO. Values represent an average (±SEM) of data obtained from 4-5 mice in each group. Values with different letter superscripts indicate significant differences at $P < 0.05$. C, control; R, rapamycin-treated; CL, crowded litter; CR, calorie-restricted.
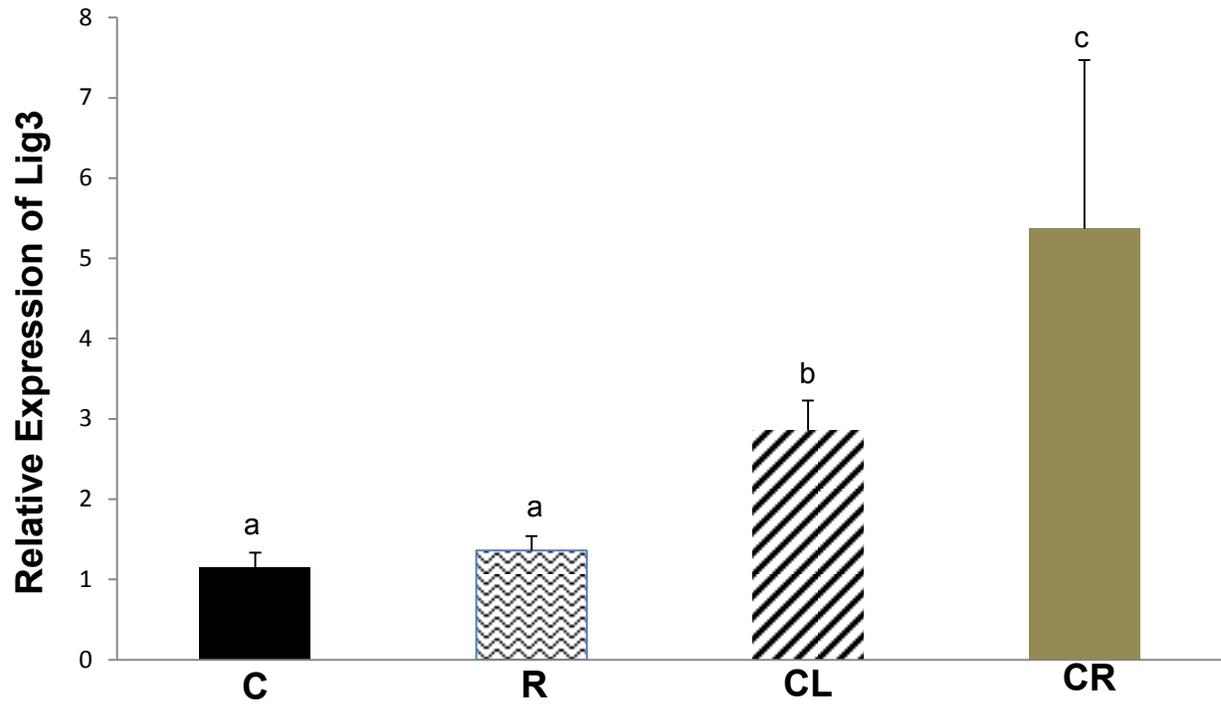
FIGURE 4-6

Figure 4-7. Impact of rapamycin diet, crowded litter, and calorie restriction on expression of GADD45 mRNA levels. GADD45 mRNA levels in the liver tissue of mice in each group were quantified using real-time PCR and normalized against RPLO. Values represent an average (±SEM) of data obtained from 4-5 mice in each group. Values with different letter superscripts indicate significant differences at $P < 0.05$. C, control; R, rapamycin-treated; CL, crowded litter; CR, calorie-restricted.
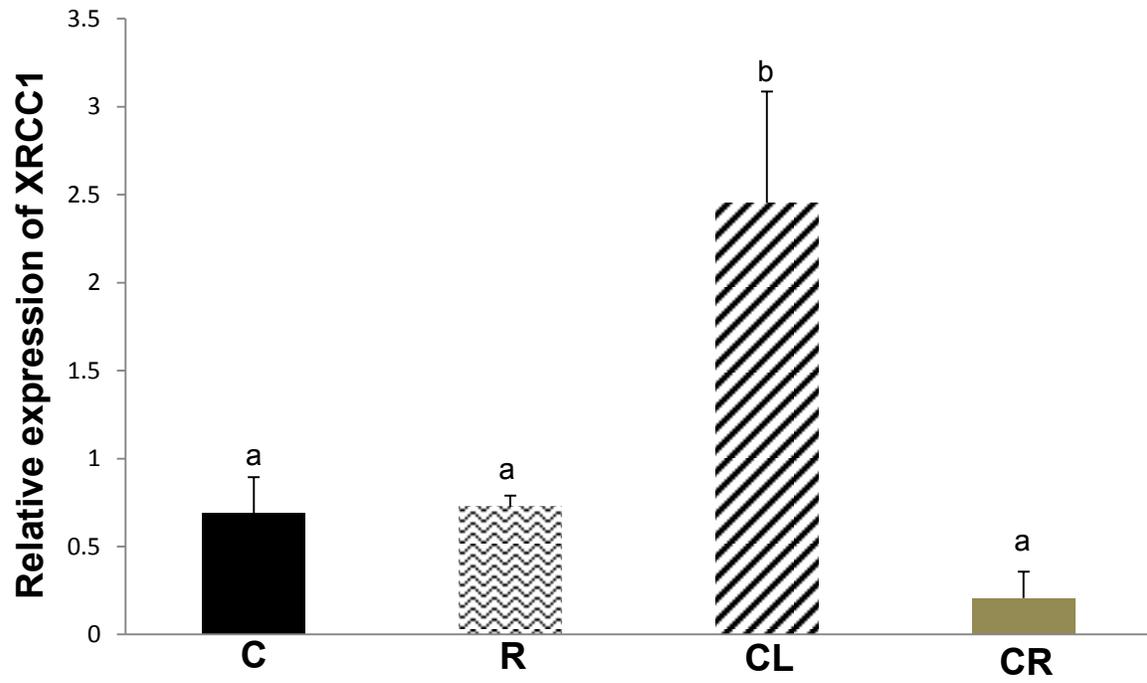
FIGURE 4-7

Figure 4-8. Impact of rapamycin diet, crowded litter, and calorie restriction on expression of PARP mRNA levels. PARP mRNA levels in the liver tissue of mice in each group were quantified using real-time PCR and normalized against RPLO. Values represent an average (±SEM) of data obtained from 4-5 mice in each group. Values with different letter superscripts indicate significant differences at $P < 0.05$. C, control; R, rapamycin-treated; CL, crowded litter; CR, calorie-restricted.
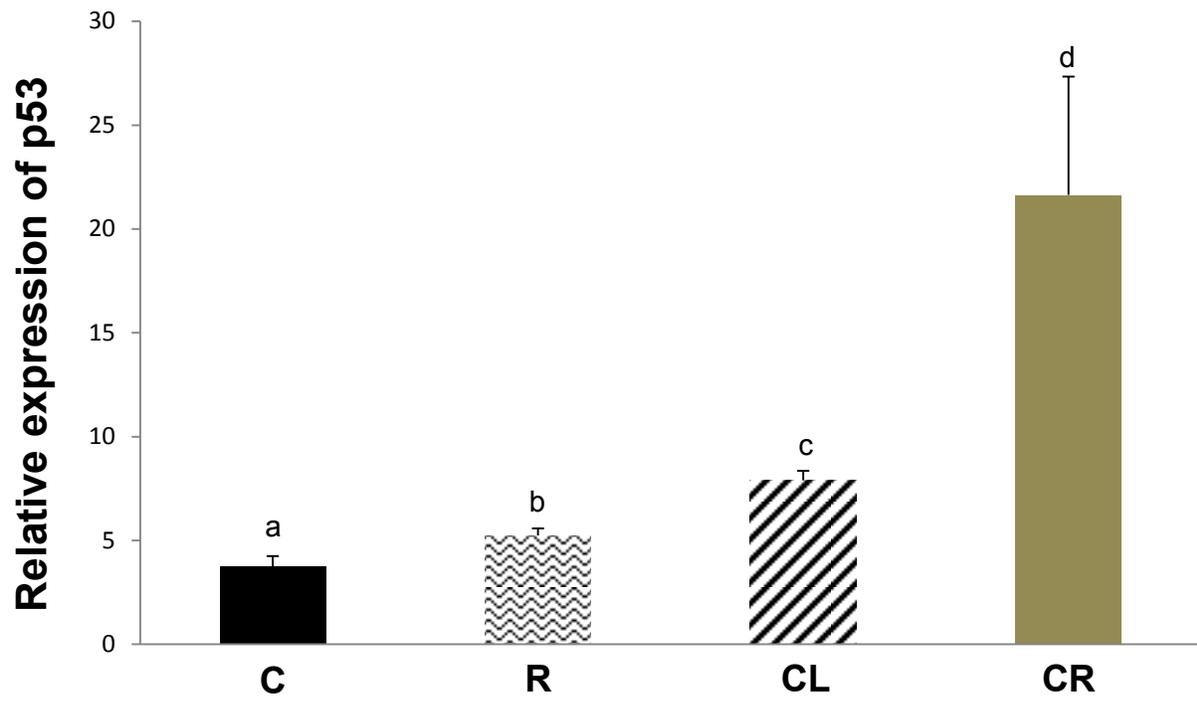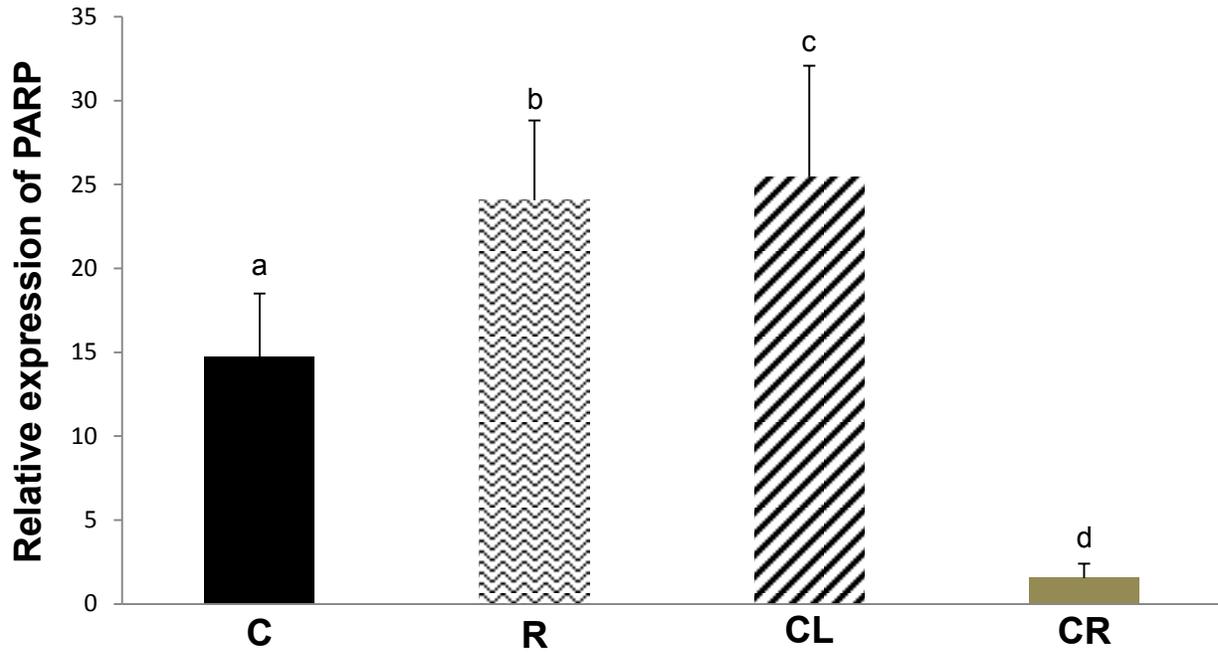
FIGURE 4-8

Figure 4-9. Impact of rapamycin diet, crowded litter, and calorie restriction on expression of MTOR mRNA levels. MTOR mRNA levels in the liver tissue of mice in each group were quantified using real-time PCR and normalized against RPLO. Values represent an average (±SEM) of data obtained from 4-5 mice in each group. Values with different letter superscripts indicate significant differences at $P < 0.05$. C, control; R, rapamycin-treated; CL, crowded litter; CR, calorie-restricted.
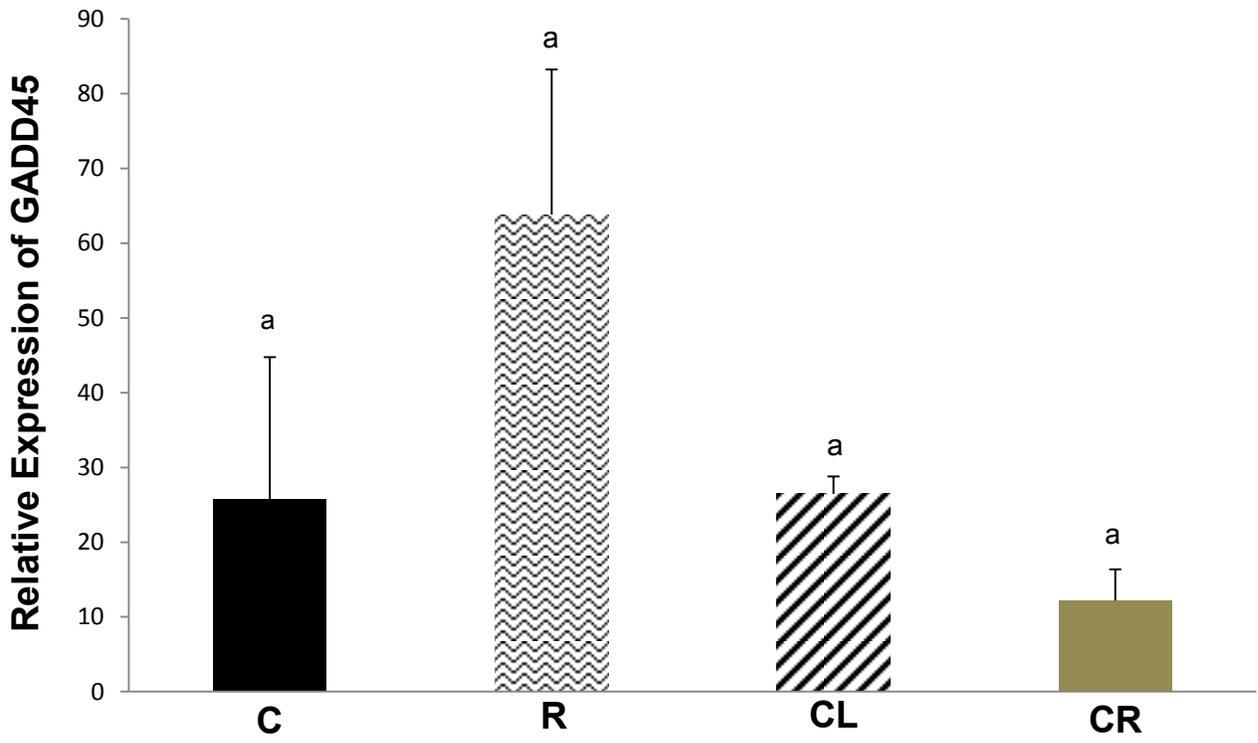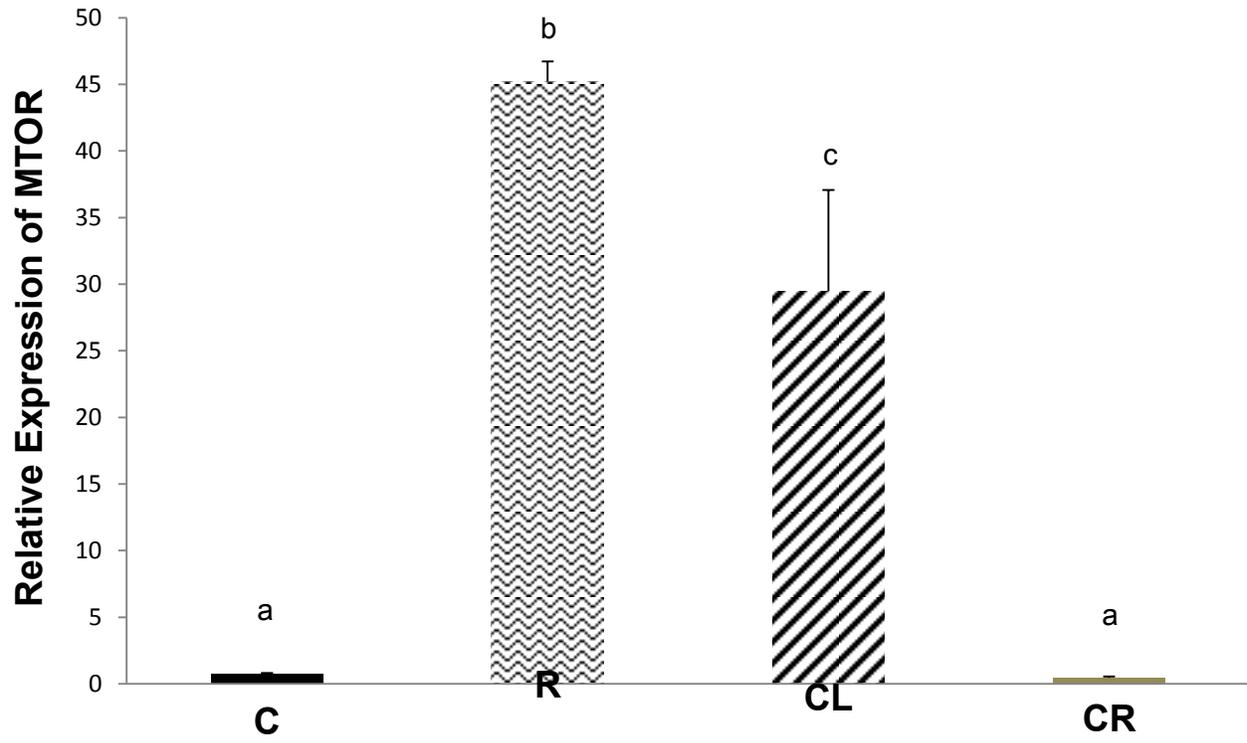
FIGURE 4-9

Figure 4-10. Impact of folate status on expression of mRNA levels in various BER genes. mRNA levels for each gene in liver tissue of mice fed a folate-adequate or folate-deficient diet were quantified using real-time PCR. UNG, OGG1 and APE1 were normalized against RPLO. β-pol, XRCC1, and LIG3 were normalized against GAPDH. Values represent an average (±SEM) of data obtained from mice in each group. Values with an asterisk indicate significant differences at $P < 0.05$. FA, folate adequate; FD, folate deficient.
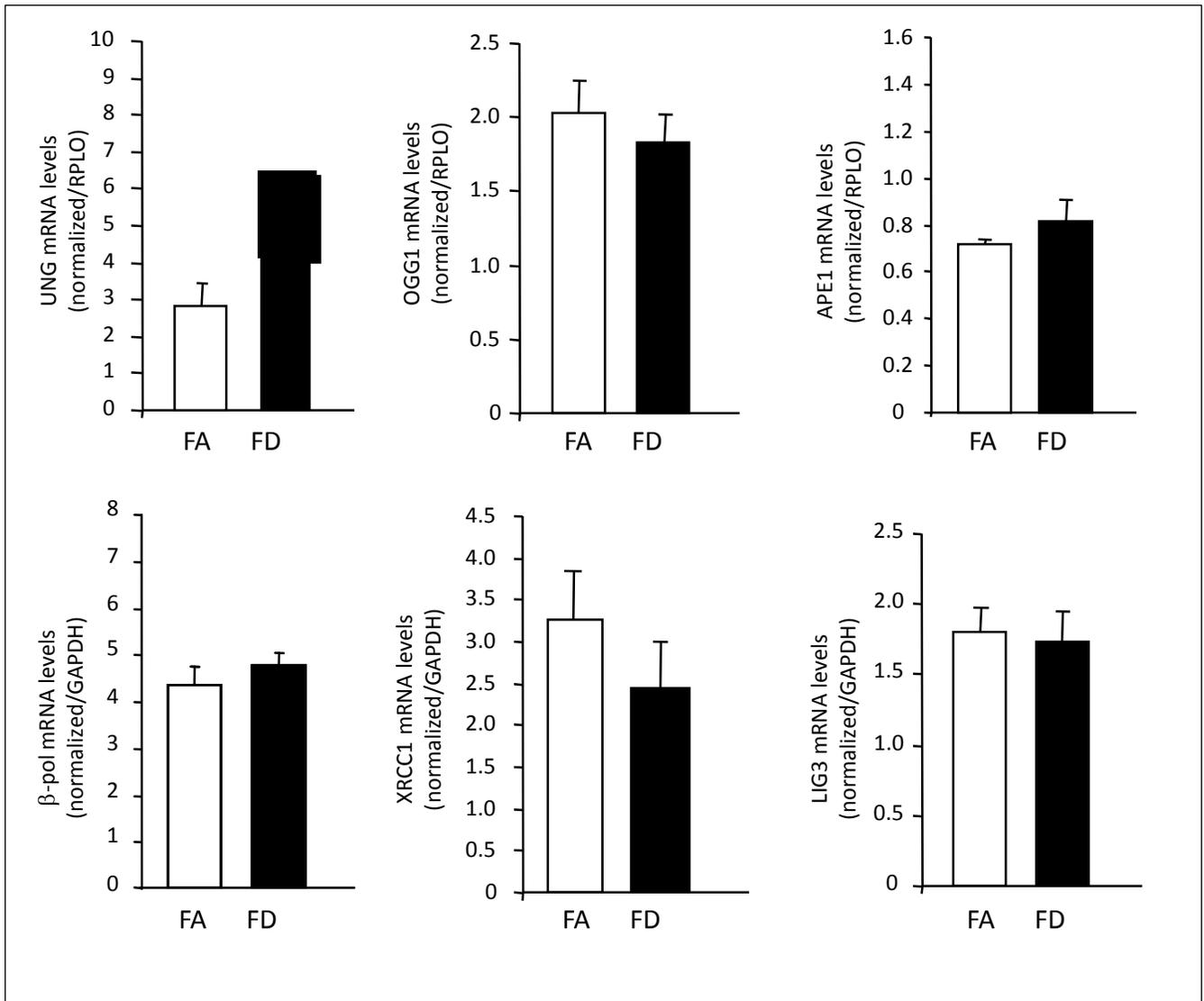
FIGURE 4-10

CHAPTER 5

RESULTS/DISCUSSION

Rapamycin, calorie restriction, and crowded litter placement have all been shown to positively influence life extension in rodents by enhancing the DNA base excision repair pathway. In this experiment, 3 different treatments were initiated in mice at 4-6 weeks of age. Mice samples were started on either a rapamycin supplemented diet, a reduced calorie diet (being provided 60% of an *ad-libitum* diet), or placed in litters consisting of 10 or more pups, during the first 3 weeks of life. mRNA levels in the liver tissue of mice in each group were quantified using real-time PCR and normalized against RPLO (ribosomal protein L15), which was used as the housekeeping gene. The following are the findings of the expression of each gene tested in response to exposure to various environments.

Uracil DNA glycosylase (UNG) is a repair enzyme that prevents mutagenesis by eliminating uracil from DNA. This is done through cleavage of the glycosidic bond and subsequently initiating the base excision repair (BER) pathway. Uracil bases are usually incorporated into DNA by cytosine deamination or mis-incorporation. As figure 4-1 shows, expression of UNG is upregulated in the rapamycin group and downregulated in the CR group. In comparison, figure 4-10 illustrates an upregulation of UNG expression in the folate-deficient (FD) group, which verifies that folate deficiency has an impact on the initiation of the BER pathway. The asterisk represents a P value < 0.05 that is statistically significant.

Apurinic endonuclease (APE) employs an important role in repair activity of the cell. The product of this APE gene is primarily responsible for the cleaving near abasic sites to yield a 3' OH adjacent to a 5' deoxyribosephosphate, in order to begin the repair of various mutations. Figure 4-2 shows APE expression is down-regulated in the CR group, but slightly upregulated in the CL group. In figure 4-10, increased expression is visible in the FD group. While there is not much evidence to show that CL may in fact extend lifespan, the increased expression of APE in the crowded litter group is an encouraging sign.

DNA polymerase β (β-pol) is the main human DNA polymerase and an integral part of the BER pathway. It has been determined that over expression of β-pol mRNA is related to a number of cancers. Therefore, expression of β-pol is very important considering its role in prevention of malignant proliferation in the cell. As observed in figure 4-3, β-pol expression is downregulated in the CR group. However, in figure 4-10, β-pol is upregulated in the FD group. As expected, BER activity was increased of genes UNG, APE, and β-pol in the folate-deficient group. The ability to respond in this manner is related to experienced stress levels.

Ligase III (Lig3) and X-ray cross-complementing gene 1 (XRCC1) are essential genes in the BER pathway. DNA ligase III, along with its cofactor XRCC1, catalyzes the nick-sealing step in short-patch BER. Expression of Lig3 is shown to be upregulated in the CR group in figure 4-4, and slightly upregulated in figure 4-10 in the folate-adequate (FA) group. Figure 4-5 shows an upregulation of XRCC1 in the CL group, and in figure 4-10, an upregulation in the folate adequate (FA) group is seen for XRCC1.

Protein 53 (p53) or tumor protein 53 is a tumor suppressor protein that is crucial in multicellular organisms, where it regulates the cell cycle and ultimately, plays a role in preventing cancer. p53 has been described as "the guardian of the genome" because of its role in conserving stability by preventing genome mutation (11, 38). The tumor suppressor p53 is activated by different types of cellular stress, including DNA damage and oncogene activation. From first glance, figure 4-6 clearly shows the highest upregulation of p53 in the CR group, strengthening the hypothesis of calorie restriction having a positive effect on cancer prevention. Different letter superscripts indicate significant differences at $P < 0.05$.

Growth arrest and DNA-damage-inducible protein gamma (Gadd45g) is a p53-regulated growth arrest and DNA-damage inducible gene that is also regulated in a p53 independent manner (54).Gadd45 genes are implicated in many basic processes shown to be intimately linked to aging and age-related diseases, including DNA repair, maintaining genome stability, epigenetic regulation, cell cycle arrest, cellular senescence, apoptosis, cell survival, inflammatory responses and immunity, and embryogenesis (4). Evidence suggests that Gadd45 expression is enhanced during apoptosis (54). In figure 4-7, expression of Gadd45 can be seen to be upregulated in the rapamycin group. In the CR group, however, Gadd45 expression is slightly reduced in comparison to the control. Although the evidence from various studies (4, 54) supports the fact that Gadd45 regulation is correlated with induction of apoptosis, it remains unclear whether this gene activates apoptosis or whether its upregulation occurs as a consequence of stress response.

Poly (ADP-ribose) polymerase (PARP) is a family of proteins involved in a number of cellular processes involving mainly DNA repair and programmed cell death. The main role is to detect and signal single-strand DNA breaks (SSB) to the enzymatic machinery involved in the repair (55). PARP activation is an immediate cellular response to metabolic, chemical, or radiation-induced DNA SSB damage. Once PARP detects a SSB, it binds to the DNA, and, after a structural change, begins the synthesis of a poly (ADP-ribose) chain (PAR) as a signal for the other DNA-repairing enzymes such as DNA Lig3, β-pol, and scaffolding proteins such as XRCC1 (55). Figure 4-8 reveals that PARP expression significantly upregulated in both the rapamycin and CL group. Although, the different letter superscripts indicate significant differences at $P < 0.05$, a definite increase in PARP expression can be seen in both groups.

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription. The TORC1 branch of the TOR pathway has been shown to be successfully inhibited pharmacologically by rapamycin, and counter DNA damage. However, figure 4-8 unmistakably shows upregulation of mTOR in the rapamycin group. mTOR activity failed to be inhibited in response to rapamycin. In comparison, mTOR expression is significantly downregulated in the CR group. A decrease in nutrients from CR may also deactivate mTOR through disabling the insulin/PI-3K pathway. Similarities between the effects of rapamycin treatment and those of a reduced-calorie diet have supported speculation that the ability of calorie-restriction to extend rodent lifespan may be mediated at least partly by downregulation of mTOR in one or more tissues (20).

CHAPTER 6

CONCLUSION

Several studies have indicated that rapamycin treatment and calorie-restricted diets have a beneficial effect on life extension in mice. Years of research have provided evidence of various treatments being utilized to delay the effects of aging and boost DNA repair activity, in order to ultimately prevent the development of tumorigenesis as well as other age related diseases. Rapamycin acts as an mTOR inhibitor which may be the key to its ability to be efficacious as a prolongevity intervention. As illustrated previously, figure 1-6 shows a reduction in oxidative damage and modulation of genomic instability, due to calorie restriction, resulting from BER activity enhancement.

Although, some of the findings from this research study resulted in conflicting results, a few positive results were also seen. The results of this investigation clearly demonstrate an upregulation of some BER genes via CR diet, and other BER genes upregulated via rapamycin treatment. Unfortunately, mTOR inhibition was not seen in rapamycin treatment. Despite little evidence to support the beneficial effects of crowded litter placement, increased activity of some BER genes was witnessed. In comparison, it is evident that a folate restricted environment reveals increased mRNA expressional levels, especially in vital BER genes (UNG, APE, and β-pol).

Aging research has entered a new era in which we are beginning to reap significant benefits that hold promise of preventing and/or delaying the severity of many age-related diseases. The results from this experiment are insufficient and not fully conclusive; therefore, there is a need for further studies to better understand the

different life-extending mechanisms. It will be exciting to see further research in regards to crowded litter and the potential role it can play in delaying aging. The data from further calorie restriction experiments will provide researchers with a deeper knowledge of the process by which CR reduces DNA damage, increases BER activity, and extending lifespan. Finally the exact mechanism of how rapamycin works to extend lifespan via mTOR inhibition is under intense investigation at several labs throughout the country. Further expansion in research of all three of these interventions will help ameliorate the understanding of life extension and cancer prevention.

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ABSTRACT

EXTENDING LIFESPAN USING VARIOUS PROLONGEVITY INTERVENTIONS AND THEIR EFFECTS ON ENHANCING DNA REPAIR ACTIVITY

by

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December 2013

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Major: Nutrition and Food Science

Degree: Masters of Science

Aging is not a disease; it causes a decrease in the physiological functions of cells, tissues, and organs. Aging has been considered as one of the biggest risk factors for the development of various diseases such as cancer, type-2 diabetes, obesity, atherosclerotic cardiovascular diseases, and neurodegeneration. Numerous studies have shown that lifespan can be extended in mice by genetic, dietary, and pharmacological interventions. A few prolongevity interventions currently being studied include: the drug rapamycin, that has been found to inhibit mTOR expression and exhibit anticancer properties; reduced caloric intake, a broadly acting dietary intervention for preventing carcinogenesis, and ultimately extending lifespan; and more recently, another promising strategy being studied is crowded litter placement in mice starting from a very young age. We hypothesize that the aforementioned interventions

will delay the effects of aging, through enhanced DNA base excision repair activity, which will lead to tumorigenesis prevention.

AUTOBIOGRAPHICAL STATEMENT

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Memberships

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